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A revised method with high yield for isolation of the labile trypsin-like enzyme from *Tenebrio molitor* larval midgut has been worked out. A technique using polyacrylamide gel electrophoresis (PAGE) that includes gelatin as a substrate was successfully employed for the separation and identification of numerous midgut proteases in Tenebrio and Tribolium. The PAGE-gelatin matrix revealed the inhibitory effect of BBI (the proteinaceous trypsin-chymotrypsin inhibitor from soybeans) on several Tribolium proteases - an effect which was not detectable in inhibition assays in solution. The isolation, characterization and kinetic properties of a chymotrypsin-like enzyme from the digestive tract of the locust

are described. Comparison of locust chymotrypsin to other chymotrypsins and the effect of inhibitors are discussed. (AU)

FINAL REPORT
(SECOND ANNUAL REPORT)

PROTEASES OF STORED PRODUCT INSECTS AND THEIR INHIBITION BY SPECIFIC PROTEASE INHIBITORS FROM SOYBEANS AND WHEAT GRAIN

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STATEMENT OF THE PROBLEM STUDIED

Specific protease inhibitors, which inhibit digestive proteases of insects, have evolved in plants. The inhibitors are of potential interest in protection of valuable crops from damage by insects. It has been the objective of the present study to isolate and characterize digestive proteases of several model insect pests and to investigate their naturally-occuring inhibitors from legume seeds and grains.

SUMMARY OF THE MOST IMPORTANT RESULTS

The proteinaceous trypsin-chymotrypsin inhibitor from soybeans has been found to strongly inhibit the trypsin and chymotrypsin-like enzymes from the digestive tract of three model insects (Tenebrio, Tribolium and Locusta) when assayed on protein substrates. The affinity of the proteinases for the inhibitors has been increased in immoblized systems (rather than in solution); this potentiated mutual accessibility seems to simulate the in vivo interaction between the insect and the raw soybean and bear relevance to the built-in defense mechanism against the insect.

The potential inhibitory effect of soybean components on insect proteases has been exemplified by the inhibitability of locust chymotrypsin by STI, the Kunitz soybean trypsin inhibitor which inhibits bovine chymotrypsin.

PUBLICATIONS

Sakal, E., Applebaum, S.W. and Birk, Y. (1988)
Purification and characterization of Locusta migratoria chymotrypsin. Int. J. Peptide Protein Res., Vol. 29, in press.

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* No charge to grant

PREFACE

The hypothesis that protein protease inhibitors of plant origin are of potential interest in protection of valuable crops from damage as a consequence of attack by insects served as a guideline throughout these studies. The investigation of the digestive proteases of several model insects is a pre-requisite for understanding the complex relationships that the insects have evolved with the plant. The information on insect proteolytic enzymes and more specifically, on insect trypsins and chymotrypsins, in comparison to the corresponding proteases from higher organisms, is essential for studying the selective, species specific, interactions of the naturally-occuring protease inhibitors with the insect proteases.

EXPERIMENTAL AND RESULTS

(1) Tenebrio molitor and Tribolium castaneum proteinases

1. a. Isolation of trypsin from Tenebrio molitor larval midguts.

In the First Annual Report we have described the extraction, separation, isolation and characterization of Tenebrio (adult) trypsin and chymotrypsin. We also showed that Tenebrio adult trypsin - in a similar manner to bovine trypsin - could be fully inhibited at a 1:1 molar ratio by the naturally-occuring proteinaceous trypsin inhibitors BBI from soybeans and CI from chickpeas when assayed on casein and on specific synthetic substrates. Our earlier experiments showed that trypsin is also a major proteinase in the alimentary system of Tenebrio molitor larvae. Further characterization of Tenebrio larval trypsin was delayed because of very low yield of active enzyme by the conventional isolation procedure on DEAE-cellulose columns. Enzyme lability seemed to be also the reason for the failure of release of active larval trypsin when isolated by affinity chromatography on immobilized BBI. In an attempt to revise the isolation procedure for Tenebrio larval trypsin we submitted the whole larval midgut enzyme solution to affinity chromatography on immobilized p-aminobenzamidine (PABA), for which Tenebrio larval trypsin showed a very low affinity when assayed in solution. Tenebrio larval trypsin was successfully retained by the PABA-Sepharose column from which it could be released in an active form by either 0.01 M acetic acid or by 4 M urea at a 50-60% yield with over 100 fold purification. Characterization and structure-function studies on this enzyme are now in progress.

1. b. Separation and identification of Tenebrio and Tribolium proteinases by the PAGE-gelatin technique

The PAGE-gelatin technique was employed to examine and compare the nature of the proteinases in midguts from larval and adults of Tenebrio molitor and Tribolium castaneum. The midguts from these species contained numerous proteinases that differed in electrophoretic mobilities in polyacrylamide gels containing gelatin

as a substrate. The bands corresponding to specific proteinases could be detected with the aid of their specific inhibitors.

Incubation of the larval midgut enzyme solution of Tenebrio molitor with excess of either synthetic or naturally occuring trypsin-and chymotrypsin-inhibitors prior to the electrophoretic process, resulted in the disappearance of several, but not all, gelatin-digesting bands. Characterization of the additional active proteinases that lack the specificities of trypsin and chymotrypsin is now in progress.

As described in the First Annual Report, Tribolium castaneum larval midgut extracts showed pronounced trypsin-and chymotrypsin-like activities when assayed on specific synthetic substrates. These activities were fully inhibited by the proteinaceous trypsin-chymotrypsin inhibitors BBI from soybeans and CI from chickpeas when assayed on low-molecular weight substrates. Inhibition was not achieved when a protein (casein) was used as the substrate in solution. However, when BBI was incorporated in the PAGE-gelatin assays of Tribolium larval midgut proteases, it strongly affected the proteolysis in at least three active bands corresponding to trypsins and chymotrypsins. It is suggested that the association between the proteinaceous proteinase inhibitors and the proteinase becomes feasible in the gel matrix. The PAGE-gelatin technique revealed also the presence of several cysteinyl proteinases which could be activated by thiol reducing agents and inhibited - by E64, a specific inhibitor of cysteinyl enzymes.

(2) Locust chymotrypsin

2. a. Purification

The detection and partial purification of a chymotrypsin-like enzyme (CTLE) from the alimentary system of the locust (Locusta migratoria migratorioides) was described in the First Annual Report. CTLE has been ultimately isolated from the digestive tract of the African migratory locust by ion-exchange chromatography on diethylaminoethyl (DEAE) cellulose followed by affinity chromatography on phenylbutylamine (PBA) Sepharose (Figure 1).

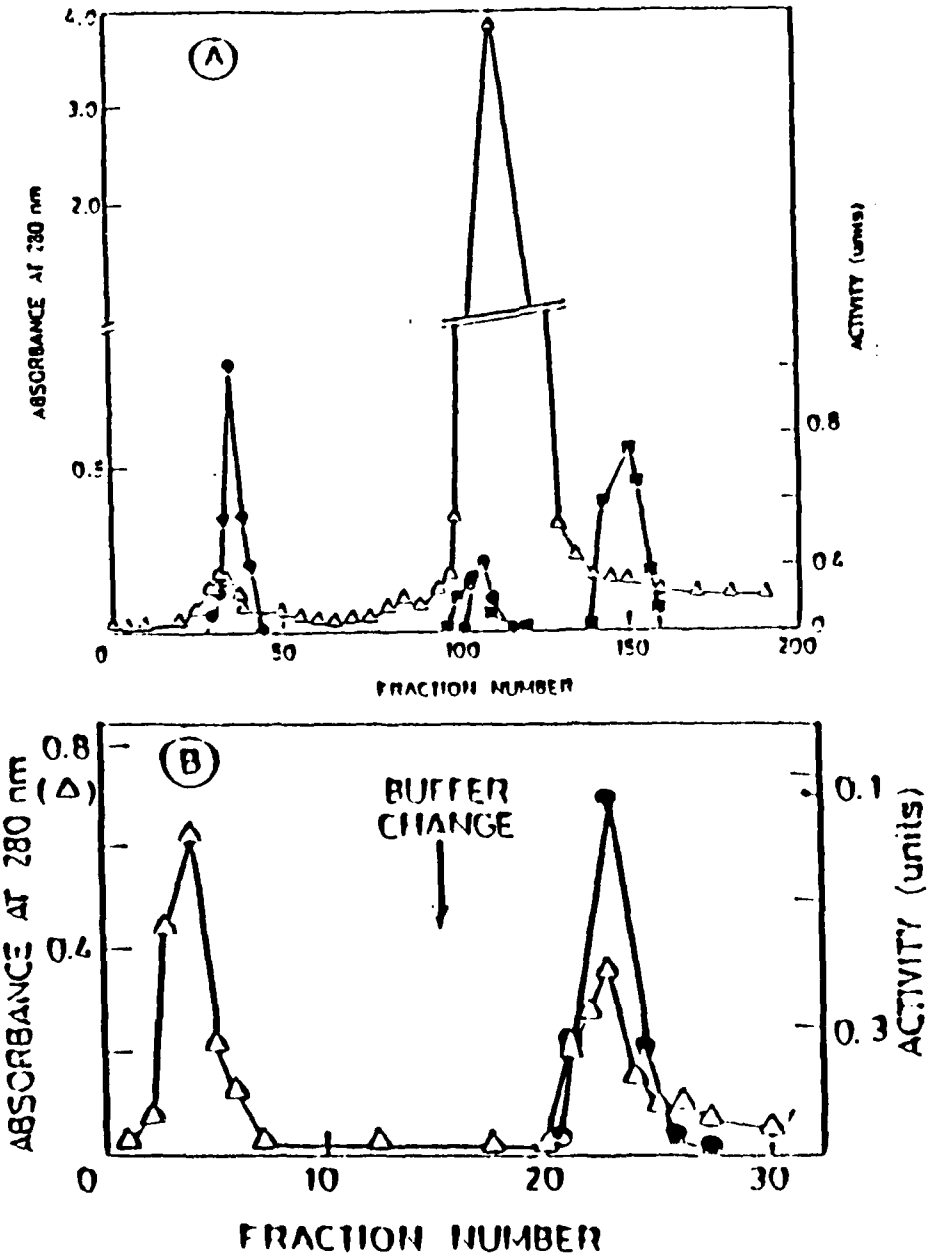

FIGURE 1
Purification of CTLE from Locusta cecae. A. Chromatography of 1 g cecal enzyme solution (CES) on a DEAE-cellulose column equilibrated with 0.01 M Tris/HCl buffer. pH 8.0. Fractions of 8.5 mL were collected. Chromatography conditions are described under Experimental Procedures and Results. Absorbance at 280nm (Δ). chymotrypic activity on ATEE (●) and trypic activity on TAME (■). B. Affinity chromatography of $CTLE_{DEAE}$ (fractions 31-49 from Fig. 1A) on a PBA-Sepharose column equilibrated with 0.01 M Tris/HCl buffer pH 8.0 containing 0.01 mM $CaCl_2$ and 0.2 M KCl. CTLE was eluted with 0.1 M PBA in the above buffer and 4-mL fractions were collected. Absorbance at 280 nm (Δ) and chymotrypic activity (●).

TABLE 1

Amino acid composition of CTLE compared to chymotrypsins from different sources[a]

Amino acid	Chymotrypsin						
	CTLE	b	c	d	e	f	g
Asp + Asn	20	15	20	15	23	23	20
Thr	20	7	10	12	8	18	23
Ser	18	10	13	14	13	24	28
Glu + Gln	20	10	13	22	11	14	15
Pro	6	7	11	10	7	12	9
Gly	26	14	15	21	16	22	23
Ala	20	7	11	19	18	21	22
Half Cys	0-1	4	1	8	0	8	10
Val	16	12	11	15	11	22	23
Met	2	0	1	2	1	2	2
Ile	8	10	8	8	6	13	10
Leu	14	11	11	12	8	19	19
Tyr	5	5	3	5	5	6	4
Phe	8	3	4	7	3	4	6
His	5	4	2	3	3	6	7
Lys	15	9	16	6	7	9	14
Arg	7	6	8	7	3	9	4
Trp	2	2	ND	ND	ND	8	8
Total	210	136	153	191	131	240	245

[a] Values are given in residues per molecule enzyme.

[b] Jany, K.D., Haug, H., Pfleiderer, G. & Ishay, J. (1978) *Biochemistry* 17, 4675–4682

[c] Golan, R. (1981) M.Sc. Thesis, Hebrew University, Jerusalem

[d] Garty, N. (1979) M.Sc. Thesis, Hebrew University, Jerusalem

[e] Birk, Y., Khalef, S. & Jibson, M.D. (1983) *Arch. Biochem. Biophys.* 225, 451–457

[f] Cohen, T., Gertler, A. & Birk, Y. (1981) *Comp. Biochem. Physiol.* 69B, 639–646

[g] Blow, D.M. (1969) *Biochem. J.* 112, 261–268

TABLE 2

N-terminal amino acid sequence of CTLE compared to chymotrypsin and trypsin from different sources

	1			5				10				15						20
a	Ile	Val	Gly	Gly	Thr	Tyr	Ala	Tyr	Ile	Ala	Gln	Tyr	Pro	Trp	Gln	Leu	X	Phe	Val	Tyr
b	Ile	Val	Gly	Gly	Thr	Asp	Ala	Pro	Arg	Gly	Lys	Tyr	Pro	Tyr	Gln	Val	Ser	Leu	Arg	Ala
c	Ile	Val	Gly	Gly	Thr	Asn	Ala	Pro	Arg	Gly	Lys	Tyr	Pro	Tyr	Gln	Val	Ser	Leu	Arg	Ala
d	Ile	Val	Gly	Gly	Val	Glu	Met	Lys	Ile	Gly	X	Phe	Pro	Trp	Glu	Ile	X	Leu	Gln	X
e	Ile	Val	Asn	Gly	Glu	Glu	Ala	Val	Pro	Gly	Ser	Trp	Pro	Trp	Gln	Val	Ser	Leu	Gln	Asp
f	Ile	Val	Gly	Gly	Tyr	Thr	Cys	Gly	Ala	Asn	Thr	Val	Pro	Tyr	Gln	Val	Ser	Leu	Asn	Ser
g	Val	Val	Gly	Gly	Thr	Arg	Ala	Ala	Gln	Gly	Glu	Phe	Pro	Phe	Met	Val	Arg	Leu	Ser	Met

[a] CTLE

[b] Jany, K.D. & Haug, H. (1983) *FEBS Lett.* 158, 98–102

[c] Jany, K.D., Bekelar, K., Pfleiderer, G. and Ishay, J. (1983) *Biochem. Biophys. Res. Commun.* 110, 1–7

[d] Tong, N.T., Imhoff, J.M., Lecroisey, A. & Keil, B. (1981) *Biochim. Biophys. Acta* 658, 209–219

[e] De Haen, C., Neurath, H. & Teller, D.C. (1975) *J. Mol. Biol.* 92, 225–259

[f] Walsh, K.A. & Neurath, H. (1964) *Proc. Natl. Acad. Sci. US* 52, 884–889

[g] Olafson, R.W., Jurasek, L., Carpenter, R. & Smillie, L.B. (1975) *Biochemistry* 14, 1168–1177

2. b. Characterization

The purity and homogeneity of CTLE have been shown by SDS-PAGE and on cellulose acetate strips. The enzyme has a molecular weight of 24 000, determined by SDS-PAGE and on a Sephadex G-75 calibrated column. It has an isoelectric point of 10.1. Amino acid analysis of CTLE, compared with chymotrypsins from other sources are given in Table 1. The low content (0-1) of half cystines reveals a major difference between the locust and vertebrates chymotrypsins. Sequence analysis of the first 20 N-terminal amino acids (Table 2) has shown 25% homology with bovine chymotrypsin and 40% homology with Vespa crabo and Vespa orientalis chymotrypsins and with Hypoderma lineatum trypsin.

2. c. Kinetic properties

The optimal pH for enzyme activity and stability was in the range of 8.5-9.0. The K_m and k_{cat} values, determined on substrates for proteolytic, esterolytic and amidolytic activity [casein, N-acetyl-L-tyrosine ethylester (ATEE) and N-acetyl-tyrosine-p-nitroanilide (ATPNA), respectively] were similar to those for bovine chymotrypsin (Table 3).

TABLE 3

Kinetic properties of CTLE compared to bovine chymotrypsin

Enzyme	Substrate	K_m		k_{cat}		k_{cat}/K_m	
		(mM)	(%)	(sec^{-1})	(abs. at 280 nm/ $sec^{-1} \times 10^{-5}$)	(sec^{-1}/mM)	(abs. at 280 nm $sec^{-1} \times 10^{-5}$)
CTLE	ATEE	1.66		58		45	
Chymotrypsin	ATEE	1.49		69		46	
CTLE	ATPNA	0.78		0.20		0.25	
Chymotrypsin	ATPNA	0.80		0.32		0.4	
CTLE	Casein		0.33		13.6		7.2
Chymotrypsin	Casein		0.35		7.0		5.8

2. d. Effect of inhibitors

CTLE was inactivated by phenyl methylsulfonyl fluoride (PMSF) and tosyl-L-phenylalanine chloromethyl ketone (TPCK), indicating the involvement of serine and histidine in its active site. The enzyme was fully inhibited by the proteinaceous, double-headed, chymotrypsin-trypsin inhibitors BBI from soybeans and CI from chickpeas, by chicken ovomucoid (COM) and turkey ovomucoid (TOM), as well as by the Kunitz soybean trypsin inhibitor (STI) that hardly inhibits bovine chymotrypsin (Figure 2). Inhibition studies of CTLE with amino acid and peptide-chloromethylketones point towards the existence of an extended binding site.

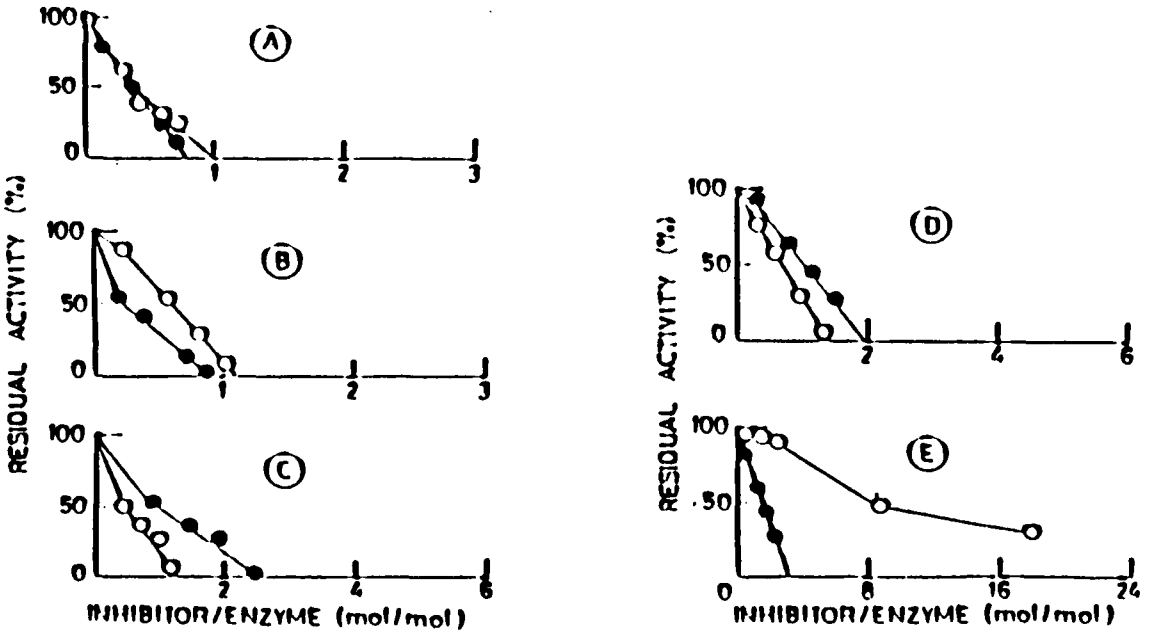

FIGURE 2.
Inhibition of CTLE (●) and bovine chymotrypsin (O) by 5 proteinaceous inhibitors. The residual activity is plotted against the molar ratio inhibitor: enzyme. (A) BBI, (B) CI, (C) COM, (D) TOM, and (E) STI.

(3) Insect Proteinase inhibitors from soybeans

In view of our recent findings by PAGE-gelatin that BBI, the proteinaceous trypsin-chymotrypsin inhibitor from soybean does inhibit the proteolytic activity of several Tribolium proteinases on protein substrates, we have focused our studies on Enzyme-Inhibitor interactions under different experimental conditions. In addition, efforts have been made to isolate, from soybeans, the highly labile protein fraction which does not inhibit trypsin or chymotrypsin but affects strongly the Tribolium cysteinyl proteases. The closer association between the proteases and their inhibitors within an immobilized matrix may simulate the in vivo interaction between the insect's digestive system and raw soybean meal.